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# Contributions of CTCF and DNA Methyltransferases DNMT1 and DNMT3B to Epstein-Barr Virus Restricted Latency

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**Establishment of persistent Epstein-Barr virus (EBV) infection requires transition from a program of full viral latency gene expression (latency III) to one that is highly restricted (latency I and 0) within memory B lymphocytes. It is well established that DNA methylation plays a critical role in EBV gene silencing, and recently the chromatin boundary protein CTCF has been implicated as a pivotal regulator of latency via its binding to several loci within the EBV genome. One notable site is upstream of the common EBNA gene promoter Cp, at which CTCF may act as an enhancer-blocking factor to initiate and maintain silencing of EBNA gene transcription. It was previously suggested that increased expression of CTCF may underlie its potential to promote restricted latency, and here we also noted elevated levels of DNA methyltransferase 1 (DNMT1) and DNMT3B associated with latency I. Within B-cell lines that maintain latency I, however, stable knockdown of CTCF, DNMT1, or DNMT3B or of DNMT1 and DNMT3B in combination did not result in activation of latency III protein expression or EBNA gene transcription, nor did knockdown of DNMTs significantly alter CpG methylation within Cp. Thus, differential expression of CTCF and DNMT1 and -3B is not critical for maintenance of restricted latency. Finally, mutant EBV lacking the Cp CTCF binding site exhibited sustained Cp activity relative to wild-type EBV in a recently developed B-cell superinfection model but ultimately was able to transition to latency I, suggesting that CTCF contributes to but is not necessarily essential for the establishment of restricted latency.**

Epstein-Barr virus (EBV) establishes a lifelong, largely quiescent (latent) infection within B lymphocytes of its human host. This requires the concerted actions of the viral latency-associated genes, several of which are believed to facilitate a germinal center (GC)-like reaction to promote differentiation of infected B cells into ones phenotypically defined as memory B cells and which serve as the primary reservoir of EBV within persistently infected individuals (reviewed in reference 59). During the establishment of latency *in vivo*, infected B cells must transition through several programs of EBV latency gene transcription, beginning with expression of the full complement of latency proteins (the latency III program), i.e., six nuclear antigens (EBNAs) and three integral plasma membrane proteins (LMPs), that is associated with a rapid EBV-induced expansion of infected cells. Thereafter, expression proceeds through a more restricted program limited to EBNA1, LMP1, and LMP2 (latency II) and ultimately to a complete restriction of EBV protein expression in the memory B cell (latency 0 [alternatively, the latency program]) (reviewed in reference 44). During subsequent periods of limited cell division, reactivation of expression of the EBV genome-maintenance protein EBNA1 alone (latency I) occurs to ensure against loss of the episomal viral genome (12).

With the exception of latency 0, each of the viral latency programs was originally described in the context of various EBV-infected cell lines or tumors (44). The great efficiency with which EBV is able to infect and immortalize primary B lymphocytes *in vitro* into lymphoblastoid cell lines (LCLs) (39) that maintain latency III has greatly facilitated our understanding of the transcriptional regulatory mechanisms involved in the early stages of establishment of EBV latency within the B-cell pool. Upon infection, transcription of the EBV genome initiates from a B-cell-specific promoter, Wp, that gives rise to the mRNAs encoding the EBNAs as well as to early latency-specific transcripts encoding the EBV Bcl-2 homolog BHRF1 (2, 4, 22, 25, 60, 61, 69). Shortly thereafter, Wp is downregulated, primarily by transcriptional interference upon EBNA2-mediated activation of the promoter Cp (~3 kbp upstream of Wp), which then becomes the dominant source of mRNAs encoding the six EBNA proteins (19, 40, 41, 49, 55, 67, 69, 70). LMP gene transcription is largely dependent on the EBNAs (1, 3, 10, 18, 33, 65, 73, 74), and therefore LMP expression follows that of the EBNAs (2). Much less is known about the transition from latency III to the restricted latency programs, as primary B cells infected with EBV *in vitro* are most likely incapable of autonomous transition to restricted latency, and their survival is dependent on maintenance of the latency III program. Consequently, the events mediating the transition to and maintenance of the restricted latency programs have been largely surmised from studies of tumor cell lines that maintain latency I or II and whose survival and growth *in vitro* are not absolutely dependent on EBV.

Nonetheless, we have a reasonably good understanding of the general process that leads to persistent EBV latency in B lymphocytes. What remains unclear, however, are the molecular mechanisms that orchestrate this process, particularly those that initiate and maintain silencing of the appropriate latency genes. Most attention in this area has focused on the role of DNA methylation, with early studies revealing the EBV genome to be progressively methylated following infection of primary B cells (24) and that

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reversal of CpG DNA methylation by treatment with 5-azacytidine results in the reactivation of EBNA and LMP expression in Burkitt lymphoma (BL) cell lines, which normally limit expression to EBNA1 (latency I) via an EBNA1-exclusive promoter (Qp) from heavily methylated EBV genomes (32). Subsequent investigations identified methylated CpG residues within latency gene promoters that either correlated with transcriptional inactivity, inhibited transcription, or prevented binding by key transcriptional activators (17, 45, 47, 48, 52, 53, 56, 62) and that in some instances were found to be actually methylated within peripheral blood B cells isolated from healthy EBV-infected individuals (37, 46). Collectively, these studies have provided strong evidence that DNA methylation is critical to establishment of restricted latency programs, though CpG methylation has been observed to lag behind transcriptional downregulation of at least one of the EBNA gene promoters (Wp) that is silent during restricted latency (16). Thus, methylation of the EBV genome may be more critical to maintenance of a transcriptionally silent state than to its initiation, though how it may be specifically regulated has yet to be determined.

Efforts to define other mechanisms that contribute to silencing of EBV latency gene expression have recently focused on a potential regulatory role for the chromatin boundary factor CTCF, which binds to several transcriptional regulatory loci within the EBV genome in latently infected B-cell lines (5, 6, 8, 13, 58). Of particular interest is the possible contribution to silencing of the common EBNA gene promoter Cp (active only in latency III), which must occur for progression to any of the restricted latency programs (i.e., latency 0, I, or II). Notably, Chau et al. (6) have provided several lines of evidence to suggest that CTCF, acting in its capacity as an enhancer-blocking factor, may promote the initiation and maintenance of restricted latency when bound to a site (−822 to −776) between Cp and its EBNA1-dependent enhancer located upstream within the EBV latency-specific origin of DNA replication, *oriP*. Further, their observation that CTCF expression and occupancy of these sites within Cp are higher in B-cell lines that maintain latency I than in those that support latency III led to the proposal that the basis for a differential silencing of Cp by CTCF might lie in its increased expression within cells able to support conversion to a restricted latency program (6). In contrast, a second study, employing a broader panel of EBV-positive cell lines, failed to note a strict correlation between CTCF binding and Cp inactivity (51). Furthermore, due to the nature of the assays performed in the former study (6), it is difficult to conclude with certainty whether CTCF is truly required for actual establishment or maintenance of restricted latency, and a subsequent analysis found that CTCF binding sites placed upstream of Cp, albeit within the context of a heterologous reporter plasmid, actually promoted EBNA1 transactivation of Cp (8). Thus, the precise contribution of CTCF to silencing of Cp is unclear.

To address the issues raised above, we directly examined the requirement for CTCF in the establishment and maintenance of restricted latency and addressed the role that differential expression of the maintenance and *de novo* DNA methyltransferase 1 (DNMT1) and DNMT3B, respectively, may play in maintenance of restricted EBV latency. The latter was prompted by our observation that DNMT1 and DNMT3B mRNA expression is consistently upregulated in B-cell lines that support latency I relative to those that sustain the latency III program. In BL lines that support latency I, stable short hairpin (shRNA)-mediated knockdown of DNMT1 and DNMT3B, either individually or in combination, to levels at or below those maintained in B-cell lines that sustain latency III did not result in the reactivation of latency III protein expression, nor did it result in detectable activation of Cp (or Wp) or a significant decrease in CpG methylation within its 5′ regulatory region. Thus, elevated expression of these DNMTs would appear not to be critical for the maintenance of restricted latency in B cells. Likewise, stable knockdown of CTCF did not result in reactivation of Cp or latency III-specific EBV protein expression in BL cells that normally maintain latency I. Most importantly, however, employing a BL cell superinfection model that we have recently developed for such studies (14), a mutant recombinant EBV (rEBV) deleted for the previously identified Cp CTCF binding site exhibited delayed silencing of Cp in transitioning from latency III to I relative to infection with wild-type (wt) rEBV. Thus, our results are consistent with a role for CTCF in the establishment of restricted latency.

## MATERIALS AND METHODS

**Cell lines.** Akata (Ak-BL), Kem I, and Mutu I are EBV-positive BL cell lines that maintain a latency I program of EBV gene expression. Kem III and Mutu III are BL lines derived from the same tumor as their counterparts Kem I and Mutu I but which maintain a latency III program. EBV-negative Ak-BL cells and their reinfected counterparts that maintain latency I have been described (50). Sal, Oku, and P3HR-1 are BL lines that maintain a Wp-restricted program of EBV latency gene expression and harbor only EBV genomes that contain an 8.5-kbp (Sal), 6.7-kbp (Oku), or 6.8-kbp (P3HR-1) deletion that removes (left to right) the C-terminal coding region for EBNA-LP, the entire BYRF1 (EBNA2) and BHLF1 open reading frames (ORFs), and the leftmost lytic cycle origin of DNA replication, $oriLyt_L$ (21). LCLs TN11/10 and Ak-LCL (gifts of John Sixbey and Lindsey Hutt-Fletcher) were generated by outgrowth *in vitro* of EBV-infected peripheral blood B cells or by infection of primary B lymphocytes *in vitro* with the Akata isolate of EBV, respectively. All lymphoid cell lines were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (HyClone). The human embryonic kidney-derived cell line HEK293 was maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (HyClone), except as noted below for production of rEBV.

**Immunoblot analysis.** EBV and cellular proteins within whole-cell extracts (50 $\mu$g per sample) were detected by standard immunoblotting techniques using the following antibodies: EBNA-1, rabbit antiserum (gift of Janet Herring); EBNA2, monoclonal antibody (MAb) PE2 (71); LMP1, monoclonal antibody S12 (31); DNMT1, clone 18 (BD Transduction Laboratories); CTCF, polyclonal rabbit antiserum (Millipore); $\beta$-tubulin, H-234 (Santa Cruz); and $\beta$-actin, MAb JLA20 (Calbiochem).

**RT-PCR.** Total cellular RNA was extracted using RNA-BEE (Tel-Test) according to the manufacturer's instructions and treated with RQ1-DNase (Promega) to remove residual DNA. cDNA was generated from 2 $\mu$g total RNA in 20-$\mu$l reaction mixtures with 200 U SuperScript III reverse transcriptase (RT) (Invitrogen) according to the manufacturer's instructions, using either 0.1 pmol gene-specific primer (GSP) for EBV transcripts or 5 ng oligo$(dT)_{12-18}$ (Invitrogen) for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and DNMT3B mRNAs. Nucleotide sequences and descriptions of primers used for RT-PCR are provided in Table S1 in the supplemental material. Corresponding negative-control reactions lacked RT. Standard PCR was performed with 2 $\mu$l of cDNA synthesis reaction product with or without RT in 25-$\mu$l volumes containing 0.5 $\mu$M each primer, 0.25 mM each deoxynucleoside triphosphate (dNTP), 2 mM $MgSO_4$, 1× HiFi PCR buffer (Invitrogen), and 1 U Platinum *Taq* high-fidelity DNA polymerase (Invitrogen). PCR parameters were as follows: 3 min of denaturation at 95°C, followed by 35 cycles of 60 s at 95°C, 60 s at the annealing temperature (see Table S1 in the

supplemental material), and 60 s at 68°C, followed by a final extension for 10 min at 68°C.

For quantitative RT-PCR (qRT-PCR) of DNMT3B gene transcripts (35), 2 μl oligo(dT)-primed cDNA was used as the template in 20-μl reaction mixtures containing iQ SYBR green Supermix (Bio-Rad) and 0.5 μM each primer. Amplification was performed using a DNA Engine Opticon2 real-time PCR machine (Bio-Rad) with the following cycling parameters: 10 min of denaturation at 94°C, followed by 35 cycles of 10 s at 95°C, 20 s at 58°C, and 15 s at 72°C. Melting curve analysis (between 65°C and 95°C with fluorescence readings taken at 0.2°C increments) was used to verify PCR specificity. Quantification of GAPDH mRNA was used to normalize expression levels between samples. The mean cycle threshold ($C_T$) was determined from three independent reactions per cDNA sample, and fold differences in expression were determined using the $\Delta\Delta C_T$ method. DNMT3B gene expression in Kem III and Mutu III cells was compared, respectively, to that in the Kem I and Mutu I BL lines, whose expression levels were arbitrarily set to 1. One-sample Student *t* tests were used to determine statistical differences.

**Knockdown of gene expression.** Stable knockdown of DNMT1, DNMT3B, and CTCF expression in Kem I and Mutu I cells was accomplished using SureSilencing shRNA expression plasmids (SABiosciences) that also encode either hygromycin or puromycin resistance. Control cell lines received an shRNA expression plasmid provided by the manufacturer that does not affect cellular gene expression. Briefly, $3 \times 10^6$ cells were transfected with plasmid by Amaxa Nucleofection (Lonza) using solution V and Nucleofection program G-016. For knockdown of DNMT1 or DNMT3B (and corresponding controls), cells received 5 μg plasmid. For knockdown of CTCF, cells were transfected with 4 different shRNA plasmids (5 μg each), and corresponding control cell lines received 20 μg control shRNA plasmid. Two days after Nucleofection, cells were plated at $2 \times 10^4$ (Kem I) or $5 \times 10^3$ (Mutu I) per well in 96-well plates and placed under puromycin selection (Kem I, 200 ng/ml; Mutu I, 2 μg/ml). After expansion of drug-resistant cells, knockdown of expression was confirmed by immunoblotting for DNMT1 and CTCF or by qRT-PCR for DNMT3B isoform 3 (due to lack of quality antibody). For double knockdown of DNMT1 and DNMT3B expression, plasmids encoding DNMT1-specific shRNAs (encoding hygromycin resistance) were introduced into lines in which DNMT3B had previously been knocked down. Following combined selection under hygromycin (Kem I, 200 μg/ml; Mutu I, 400 μg/ml) and puromycin (as described above), lines in which DNMT1 expression was knocked down were identified and the knockdown of DNMT3B expression reconfirmed.

**Analysis of CpG methylation.** Pyrosequencing (7) was used to quantitatively assess CpG methylation of the Cp region of the EBV genome. Briefly, DNA was purified from cells using the QIAamp DNA Mini Kit (Qiagen), and 1 μg (per subsequent PCR amplification) was treated with sodium bisulfite using the EZ DNA Methylation Gold kit (Zymo Research). Each PCR product was generated using a two-step approach, with the second step incorporating a 3′ biotin modification. The products from two separate amplifications (Cp1 and Cp2) together spanning the Cp region of the EBV genome per cell line were then subjected to pyrosequence analysis (see Fig. S1 in the supplemental material). For the first round of PCR, bisulfite-treated DNA was amplified in 25-μl reaction mixtures containing 0.5 μM each primer, 0.25 mM each dNTP, 2 mM $MgSO_4$, 1× HiFi PCR buffer (Invitrogen) and 1 U Platinum *Taq* high-fidelity DNA polymerase (Invitrogen). Primers used to amplify Cp1 and Cp2 were 5′Cp1 (5′-AGAAATTAGTTGAGAGGTTAGTGTTT-3′) and 3′Cp1 (5′-GGGACACCGCTGATCGTTTACCCCCCCCTAATATTATTACCACTT-3′) and 5′Cp2 (5′-GTGGGAAAAAATTTATGGTTTAG-3′) and 3′Cp2 (5′-GGGACACCGCTGATCGTTTATAAAACCTTAATCCCCCCCTTA-3′); the underlined sequence in each reverse primer is complementary to the biotinylated universal reverse primer used in the second step (see below). PCR parameters were as follows: 3 min of denaturation at 94°C, then 40 cycles of 45 s at 94°C, 30 s at 55°C, and 45 s at 68°C, followed by a final extension for 5 min at 68°C. The second round of PCR was performed as described above, but using 2 μl first-round product as the template, the original corresponding forward primer (5′Cp1 or 5′Cp2), and a biotinylated universal reverse primer (5′-biotin-GGGACACCGCTGATCGTTTA-3′) that binds to the tailed first-round products. The biotinylated strands of the PCR products were purified using a Vacuum Prep Tool (Qiagen) according to the manufacturer's protocol and were used as the template in pyrosequencing reactions in the presence of 500 nM sequencing primer using a PyroMark MD pyrosequencer (Qiagen) according to the manufacturer's recommendations. As illustrated in Fig. S1 in the supplemental material, Cp1 products were sequenced with a single primer (5′-AAGATTATTAAGTTGGTGTA-3′); sequencing of Cp2 products required two separate primers (5′-AGGATTATAGTTAATAAGAG-3′ and 5′-GTGGAGTAAAGTTTAAAGTG-3′).

**Generation of rEBV.** All EBV used in this study was rEBV derived from Ak-GFP-BAC (clone 12-15), a bacterial artificial chromosome (BAC) containing the genome of the Akata isolate of EBV (20) and maintained in *Escherichia coli* strain DH10B under chloramphenicol (Cm) selection. Generation of rEBV in which the CTCF binding site between *oriP* and Cp (6) had been deleted was accomplished by recombineering in *E. coli* strain SW105 (obtained from the Mouse Cancer Genetics Program, NCI-Frederick) (see Fig. 7). The DNA-targeting construct used consisted of a tetracycline resistance gene cassette flanked by 276- and 275-bp EBV DNA homology arms that were generated by PCR from Ak-GFP-BAC and represented DNA immediately upstream and downstream of the locus to be deleted (nucleotide coordinates 10394 to 10590 of the EBV genome; accession number NC_007605.1). The upstream arm (nucleotides 10118 to 10393) was amplified with forward primer 5′-GCctcgagCAAAGCCATGAGTGAATTTGAC-3′ (lowercase, XhoI site) and reverse primer 5′-GCaagcttgcgatcgCTGCAGTGTCCCTGCTGCC-3′ (lowercase, HindIII site; underlined, AsiSI site) and following digestion was ligated between the XhoI and HindIII restriction sites of the multiple-cloning site of pBluescript II KS(+) (Stratagene), yielding ΔCTCF-pBS-US. The downstream arm (nucleotides 10591 to 10865) was generated using forward primer 5′-GCgcgatcgcAGGCCTTGCAGGGCAGAC-3′ (lowercase, AsiSI site) and reverse primer 5′-GCgaattcGCTTGGGTTTCTAATTGGGACAC-3′ (lowercase, EcoRI site), digested, and ligated into ΔCTCF-pBS-US between the AsiSI site within the upstream arm and the EcoRI site of pBluescript II KS(+), making ΔCTCF-pBS-USDS, which was verified by DNA sequence analysis. Next, an AsiSI restriction fragment containing a tetracycline resistance gene flanked by Flippase (Flp) recombinase target (FRT) sites was purified from the plasmid pFRT-rpsL-Tet-FRT (*rpsL* is a counterselection gene irrelevant to these studies) and ligated into the AsiSI site of ΔCTCF-pBS-USDS between the EBV DNA-targeting arms. From this plasmid, the DNA-targeting fragment (US-FRT-rpsL-Tet-FRT-DS) was removed by XhoI and EcoRI digestion and purified by agarose gel electrophoresis.

To generate ΔCTCF-Ak-GFP-BAC, SW105 cells containing Ak-GFP-BAC were grown at 32°C to an optical density at 600 nm ($OD_{600}$) of 0.5 and then induced to express recombination proteins by being shifted to 42°C for 15 min, followed by rapid cooling and washing (27). These cells were then transformed (by electroporation) with 300 ng targeting fragment and selected for tetracycline resistance ($Tet^r$). To remove DNA flanked by the EBV DNA-targeting arms, $Tet^r$ clones were grown at 32°C to an $OD_{600}$ of 0.5; L-arabinose was then added to 10% (wt/vol) to induce expression of the Flp, and incubation was continued for 1 h. Bacteria were then plated onto LB agar plates, and $Cm^r$ colonies were replica plated onto tetracycline-containing agar to identify those that had lost the tetracycline resistance cassette. To verify proper recombination, BAC DNA from $Cm^r$ $Tet^s$ colonies was amplified by PCR and subjected to DNA sequence analysis using primers with annealing sites in the EBV genome outside the two homology arms present in the targeting fragment. To ensure against illegitimate recombination elsewhere in the EBV genome, BAC DNA purified using the NucleoBond BAC100 kit (Clontech) was examined by agarose gel electrophoresis and standard Southern blot hybridization analysis following digestion with BamHI (see Fig. 7) or NheI (not shown).

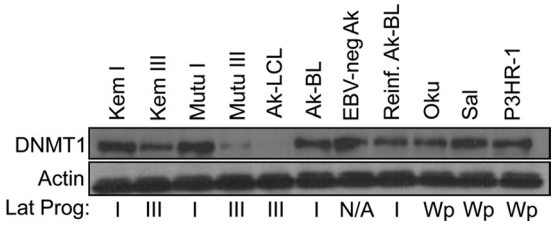


**FIG 1** DNMT1 expression is elevated during restricted EBV latency. Levels of DNMT1 within B-cell lines that maintain the EBV latency I, latency III, or Wp-restricted transcriptional program (Lat Prog) were assessed by immunoblot analysis. Ak-LCL is a B LCL generated by infection *in vitro* with the isolate of EBV obtained from the Akata BL line (Ak-BL); Reinf.Ak-BL is an EBV-negative (EBV-neg) Ak-BL cell line reinfected with a recombinant Akata isolate of EBV carrying a neomycin resistance gene in its BDLF3 gene (50). $\beta$-Actin served as a loading control. N/A, not applicable.

**Virus production and superinfection of BL cells.** To produce wt or ΔCTCF rEBV, HEK293 cells were transfected with 2 $\mu$g Ak-GFP-BAC or ΔCTCF-Ak-GFP-BAC DNA, respectively, using TransIT-293 transfection reagent (Mirus), and individual clones harboring EBV-BAC DNA were selected based on green fluorescent protein (GFP) expression and growth in standard DMEM growth medium containing 500 $\mu$g Geneticin (Cell Gro) per ml. To induce EBV replication, HEK293 clones were transiently transfected with 1 $\mu$g each of expression plasmids encoding the EBV proteins BZLF1 and BALF4; at 24 h posttransfection, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and sodium butyrate were added to the culture medium to 20 ng/ml and 1.47 mM, respectively. After 3 h, cell monolayers were rinsed and then incubated in fresh RPMI (instead of DMEM) growth medium for 3 days, after which the culture medium was clarified by low-speed centrifugation and passed through a 0.45-$\mu$m filter. Successful virus production was determined by infecting Raji BL cells using a "spin-infection" protocol in which $5 \times 10^5$ cells were mixed with 1 ml of the virus-containing HEK293 culture medium in each well of a 6-well plate and centrifuged at 200 × *g* for 1 h at 4°C. Plates were then incubated at 37°C for 24 h, followed by addition of 2 ml fresh growth medium. At 3 days postinfection, Raji cells were microscopically scored for GFP expression to identify the HEK293 clones that most efficiently produced rEBV. Virus produced in this manner was used to superinfect Kem I BL cells by the same method. At 5 to 7 days postsuperinfection, cells were placed under Geneticin selection (400 $\mu$g/ml) and expanded directly from the 6-well plates.

## RESULTS

**Elevated expression of maintenance and *de novo* DNMTs during restricted latency.** That the elevated expression of DNMTs may contribute to maintenance of restricted EBV latency was suggested by a series of mRNA profiling analyses we performed to identify cellular genes differentially regulated between the latency I and III programs of EBV latency, e.g., as between the Kem I and Kem III BL lines, respectively. Our results indicated that the levels of the mRNAs encoding the maintenance and *de novo* DNA methyltransferases DNMT1 and DNMT3B, respectively, were consistently higher by severalfold in the Kem I cells. We did not detect a significant difference in the mRNA levels for DNMT3A, the second known *de novo* DNMT. For DNMT1 this difference was confirmed by immunoblotting and extended to other B-cell lines latently infected with EBV. As shown in Fig. 1, the DNMT1 level was higher in all BL cell lines that maintain latency I than in lines that support latency III, with the lowest level of DNMT1 expression observed in the one LCL examined (Ak-LCL). We observed intermediate to high DNMT1 levels (relative to Kem I) in the three lines evaluated that maintain a Wp-restricted latency (Fig. 1, Oku, Sal, and P3HR-1). Further, because DNMT1 levels were equivalent in EBV-positive and -negative Ak-BL cells and did not increase upon reestablishment of latency I after reinfection of EBV-negative Akata cells (Fig. 1), it would appear that the virus is not responsible for the apparent upregulation of DNMT1 expression during the latency I program.

We were unable to reliably assess DNMT3B levels by immunoblotting due to lack of a quality antibody to detect endogenous DNMT3B in our cell lines. Therefore, we assessed DNMT3B3 levels by qRT-PCR. As shown in Fig. 2, the levels of this DNMT3B mRNA were approximately 10-fold higher in the Kem I and Mutu I BL lines than in their isogenetic latency III-maintaining counterparts.

**Elevated expression of DNMT1 or DNMT3B alone is not required to maintain restricted latency.** We next asked whether elevated DNMT1 or DNMT3B expression is necessary to sustain the latency I program within BL cell lines, which are the most widely used *in vitro* model of a restricted EBV latency program observed in normal latently infected B cells within the host. To do this, we introduced shRNA expression vectors to stably knock down DNMT1 or DNMT3B in Kem I and Mutu I BL cells and assessed whether this would result in reactivation of the latency III program. As shown in Fig. 3A, we achieved stable knockdown of DNMT1 to levels approximating or even below that seen in the respective Kem and Mutu BL lines that maintain latency III. However, in none of these cell lines did we observe an activation of either EBNA2 or LMP1 expression indicative of latency III (Fig. 3B). We did observe an apparent increase in the expression of

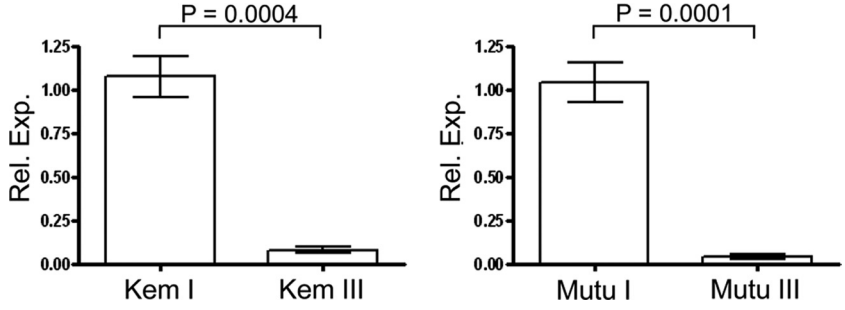


**FIG 2** *DNMT3B* expression is elevated during restricted EBV latency. The relative levels of the mRNAs encoding the *de novo* DNA methyltransferase DNMT3B during latency I and III maintained within paired Kem I/III and Mutu I/III BL cell lines were measured by qRT-PCR. Data shown are for the mRNA isoform 3B3 encoding DNMT3B (35) and are from a representative experiment in which each RNA/cDNA sample was analyzed in triplicate. GAPDH mRNA levels were used to normalize input between samples. The one-sample Student *t* test was used to determine statistical differences.

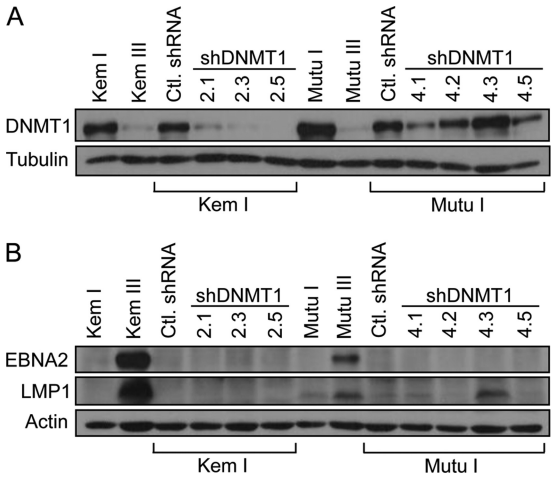


**FIG 3** Reduction of DNMT1 levels in BL cells maintaining latency I does not result in reactivation of the latency III program. (A) Immunoblot analysis of DNMT1 expression in Kem I and Mutu I BL cell lines stably expressing control (Ctl.) or DNMT1-specific (shDNMT1) shRNAs. (B) EBV EBNA2 and LMP1 expression were assessed as markers of potential reactivation of the latency III program in the cell lines analyzed in panel A. Immunoblot detection of $\beta$-tubulin and $\beta$-actin served as loading controls in panels A and B, respectively.

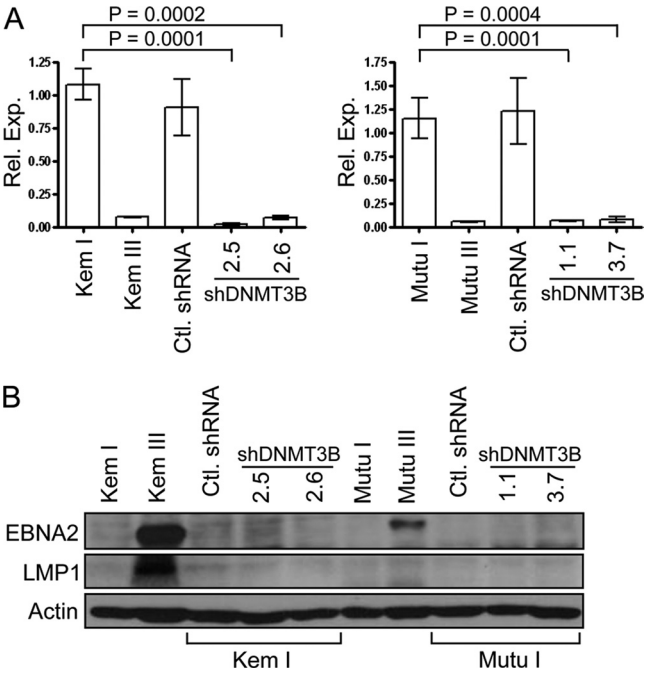


**FIG 4** Reduction of *DNMT3B* levels in BL cells maintaining latency I does not result in reactivation of the latency III program. (A) qRT-PCR analysis of *DNMT3B* in Kem I (left panel) and Mutu I (right panel) BL cell lines stably expressing control (Ctl.) or DNMT3B-specific (shDNMT3B) shRNAs. (B) EBV EBNA2 and LMP1 expression was assessed as markers of potential reactivation of the latency III program in the cell lines analyzed in panel A. Immunoblot detection of $\beta$-actin served as a loading control. The one-sample Student *t* test was used to determine statistical differences.

LMP1 in one Mutu I line (Fig. 3B, shDNMT1 line 4.3), but knockdown of DNMT1 in this line was minimal, if at all, and the DNMT1 level was actually higher than in the negative-control line, which, like line 4.1 (with the greatest degree of knockdown), expressed a low level of LMP1 comparable to that seen in the parental Mutu I cells. Further, unlike Kem I cells, which stringently maintain a latency I pattern of EBV gene expression, we often observe a low level of LMP1 expression in Mutu I cells, suggesting that a subpopulation of these drift between latency I and II. Thus, it is unlikely that any expression of LMP1 in these Mutu I-derived lines was due to specific effects of the DNMT1 shRNA.

We next performed analogous experiments to determine whether knockdown of DNMT3B was sufficient to promote reactivation of latency III. Again, we assessed DNMT3B expression by qRT-PCR. Note that while we assessed our knockdown efficiency by determining the level of the 3B3 isoform of DNMT3B mRNAs (35), the shRNAs employed were designed to target all of the DNMT3B-coding mRNAs. As shown in Fig. 4A, we achieved significant and stable knockdown in two lines each of Kem I and Mutu I cells to levels at or below those maintained in the respective Kem III and Mutu III lines. As we had observed for DNMT1, knockdown of DNMT3B did not result in the activation of either EBNA2 or LMP1 expression (Fig. 4B).

**Combined elevation of DNMT1 and DNMT3B is not required for maintenance of restricted latency.** Although DNMT1 is considered the cellular maintenance DNMT, knockout of both DNMT1 and DNMT3B alleles by homologous recombination in cultured colorectal tumor cells has been shown to result in a minimal decrease in global DNA methylation, whereas genetic inactivation of both DNMTs together results in nearly complete loss of genome methylation (43). Therefore, to determine whether elevated expression of both DNMT1 and DNMT3B is required to sustain restricted latency, we performed double DNMT knockdown by targeting DNMT1 expression within our Kem I and Mutu I BL lines in which DNMT3B had previously been targeted. As demonstrated in Fig. 5A, DNMT1 expression was reduced to levels closely approximating the lower levels seen in the respective Kem III and Mutu III cell lines. Reevaluation of these cells for *DNMT3B* expression confirmed that it had not been restored (data not shown). However, as we had observed for Kem I and Mutu I cells in which DNMT1 and DNMT3B had been singly targeted, we did not observe a resulting activation of EBNA2 or LMP1 expression (Fig. 5A). An RT-PCR-based analysis of EBNA promoter usage, furthermore, revealed that neither Cp nor Wp had been activated and that Qp, the promoter responsible for EBNA1 expression during latency I (34, 54), remained the source of EBNA1 mRNA (Fig. 5B).

Finally, to determine whether knockdown of either or both DNMTs had any influence on the methylation status of Cp, we subjected DNA isolated from parental, control, and the single and double DNMT knockdown lines to methyl-CpG analysis by sodium bisulfite treatment and pyrosequencing (7). The results from this analysis indicated that there had not been a significant decrease in CpG methylation within Cp as a consequence of reduced DNMT expression (see Fig. S1 in the supplemental material), consistent with our inability to amplify transcripts indicative of Cp reactivation (Fig. 5B). Thus, despite the consistent elevation of DNMT1 and DNMT3B expression among BL lines that retain the latency I program relative to those that support latency III, this is unlikely to be a mechanism for the maintenance of restricted latency, at least within the context of the BL model of restricted latency.

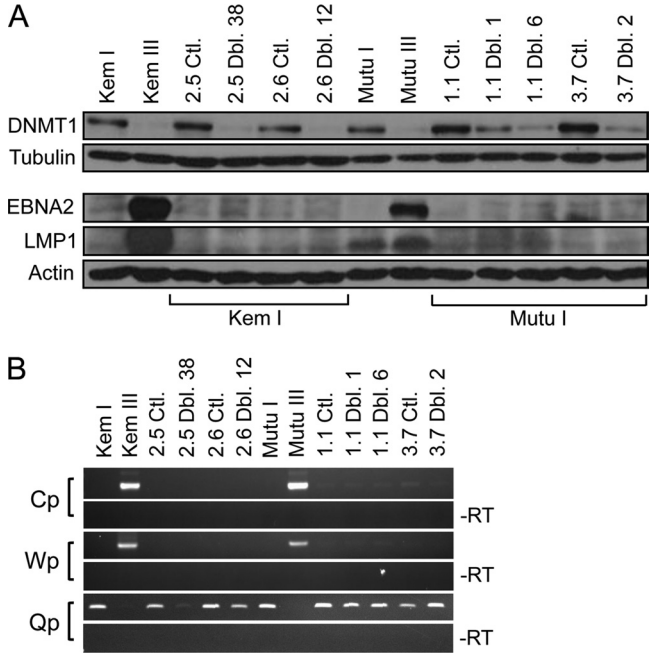


**FIG 5** Combined knockdown of DNMT1 and DNMT3B does not reactivate latency III-associated mRNA and protein expression. (A) Demonstration by immunoblotting of the knockdown of DNMT1 within Kem I and Mutu I BL cell lines in which stable knockdown of *DNMT3B* had been previously achieved (Fig. 4), i.e., double knockdown (Dbl). Control (Ctl.) lines expressed the standard control (non-DNMT-specific) shRNA in addition to the DNMT3B-specific shRNA. EBV EBNA2 and LMP1 expression was assessed as markers of potential reactivation of the latency III program; immunoblot detection of β-tubulin and β-actin served as loading controls. (B) Lack of detection by RT-PCR of mRNAs from the latency III-specific *EBNA* promoters Cp and Wp indicates that transcriptional silencing of these promoters is sustained upon combined knockdown of DNMT1 and *DNMT3B*. Kem III and Mutu III served as positive controls for the detection of Cp and Wp usage; detection of Qp-specific EBNA1 mRNAs expressed during latency I in Kem I and Mutu I cells and their derivative cell lines served as a positive control for RNA integrity. Note that a faint larger cDNA amplified with Cp- and Wp-specific primers is the result of retention of the 81-bp intron between exons W1/W01 and W2. −RT, absence of reverse transcriptase in the cDNA synthesis reaction mixture prior to amplification by PCR.

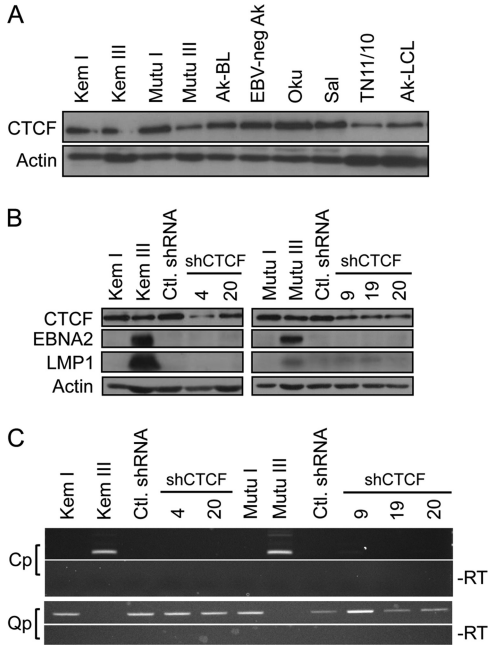


**FIG 6** Elevated CTCF expression is not essential for maintenance of restricted EBV latency. (A) CTCF and β-actin (loading control) were detected by immunoblotting in B-cell lines maintaining the latency I (Kem I, Mutu I, and Ak-BL), latency III (Kem III, Mutu III, LCL TN11/10, and Ak-LCL), or Wp-restricted (Oku and Sal) program of EBV latency and in EBV-negative Akata BL cells (EBV-neg Ak). (B) Stable shRNA-mediated knockdown of CTCF in Kem I (left panel) and Mutu I (right panel) BL cells maintaining latency I to levels at or below those observed within cells maintaining latency III (Kem III and Mutu III) did not result in reactivation of EBNA2 and LMP1 expression characteristic of latency III. (C) Transcriptional silencing of the latency III-specific EBNA promoter Cp is sustained upon stable knockdown of CTCF. RNA isolated from the cells analyzed in panel B was subjected to RT-PCR to detect Cp (latency III) and Qp (latency I). −RT, absence of reverse transcriptase in the cDNA synthesis reaction mixture prior to amplification by PCR.

**Role of CTCF in maintenance of restricted latency.** Beyond CpG methylation within latency gene promoters, one study has suggested that upregulated expression of CTCF may be pivotal to establishment and maintenance of restricted programs of EBV latency, possibly via an enhancer-blocking function when bound between Cp and its EBNA1-dependent enhancer within *oriP* (6). However, while CTCF was shown to have a negative effect on EBNA2 mRNA expression via its binding site in Cp, whether CTCF is truly essential for the establishment or maintenance of restricted latency was not directly addressed (see below also). A subsequent study, furthermore, failed to note a strict correlation between CTCF occupancy and Cp inactivity (51).

To establish whether elevated CTCF expression is indeed involved in the maintenance of restricted latency, we first assessed CTCF levels within two sets of paired BL cell lines (Kem I/III and Mutu I/III), with both lines of each set having originated from the same tumor but which maintain either latency I or III. As each set is in theory isogenic, they represent ideal models with which to delineate the influence of CTCF on this particular aspect of EBV latency. As shown in Fig. 6A, CTCF was indeed expressed at higher levels in the BL lines Kem I, Mutu I, and Ak-BL (all of which maintain latency I) than it was within LCLs (TN11/10 and Ak-LCL), consistent with the previous report (6). Note that although the relatively higher levels of β-actin (loading control) detected within the LCL samples suggest that these lanes had been overloaded, indicating that CTCF levels may be even lower in the LCLs, we have found when comparing LCL to BL cell lysates, either from equal cell number or containing equal total protein, that the β-actin level is consistently higher within LCLs. Thus, the CTCF levels detected in LCLs as shown in Fig. 6A (without correction for apparent overloading) are likely a true representation of the actual amount of this protein within LCLs relative to BL cells. Interestingly, though CTCF levels were consistently higher in Kem I and Mutu I cells than in their latency III counterparts (Fig. 6A and B), the differences in CTCF expression between latency I and III within these isogenetic pairs of BL lines were modest and clearly not as great as those between the latency I BL lines and LCLs as shown here and noted previously (6). Also, CTCF levels were equivalent in EBV-negative and EBV-positive Ak-BL cells, suggesting that the virus is not responsible, directly or indirectly, for promoting CTCF expression. Finally, we noted that CTCF levels were also relatively high within the BL lines Oku and Sal, which maintain the variant Wp-restricted

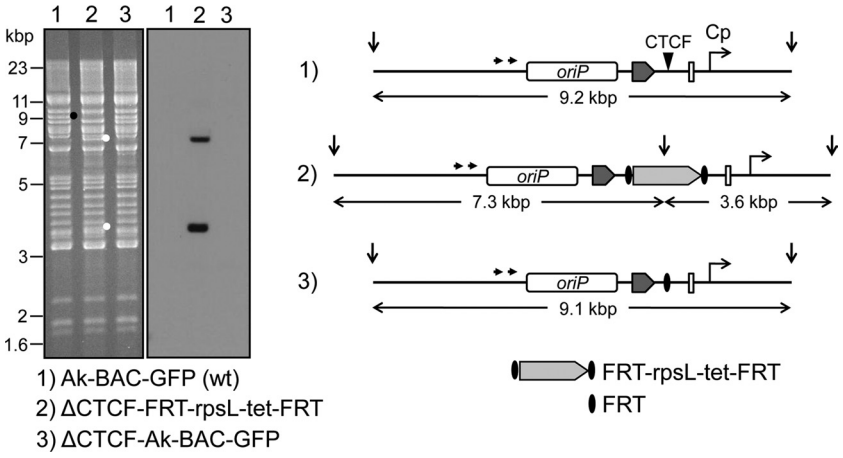


**FIG 7** Generation of ΔCTCF rEBV. Shown is the recombineering strategy used to generate a mutant rEBV genome deleted for the previously identified CTCF binding site within Cp (6). (Left) Agarose gel electrophoresis of BamHI-digested and corresponding Southern blot of parental Ak-BAC-GFP DNA (lane 1), the intermediate BAC clone containing the targeting fragment with the cassette FRT-rpsL-tet-FRT in place of the CTCF binding site in the BamHI-C fragment of the EBV genome (lane 2), and the final BAC clone of ΔCTCF rEBV after Flp-mediated removal of the targeting cassette (lane 3). The black dot denotes the BamHI-C restriction fragment from the BAC clone of the Akata EBV genome (lane 1) and white dots the expected BamHI restriction fragments after insertion of the targeting cassette with a single BamHI restriction site in the tetracycline resistance gene into BamHI-C, resulting in loss of the BamHI-C fragment upon digestion (lane 2). The Southern blot was probed with $^{32}$P-labeled selection cassette fragment to ensure that inappropriate recombination had not occurred outside the desired locus. (Right) Configuration of the BamHI-C locus within the BAC clone of the Akata EBV genome is shown above the intermediate and final configurations of the locus during recombineering to delete the CTCF binding site upstream of Cp. The locations within BamHI-C of the Pol III genes encoding the EBER1 and EBER2 transcripts (small arrows), the latency origin of DNA replication *oriP*, the lytic cycle gene *BCRF1* (dark block arrow), the CTCF binding locus, the EBNA2-responsive enhancer (open rectangle), and the Cp transcription start site (bent arrow) are indicated for reference. Vertical arrows indicate locations of BamHI restriction sites; BamHI restriction fragments denoted in lanes 1 and 2 in the ethidium bromide-stained agarose gel (left) are shown under the respective DNA configuration (right). After Flp-mediated removal of the FRT-flanked selection cassette, a single FRT site remains at the site of the 197-bp deletion that removed the CTCF binding site, resulting in a slightly smaller BamHI-C fragment.

latency program. Although Cp is silent in these cells, presumably due to loss of EBNA2 expression as a consequence of a deletion common to BL cells that exhibit Wp-restricted latency (23), expression of the remaining five EBNAs is sustained via Wp (21), suggesting that any negative influence of CTCF on Cp does not necessarily extend to Wp.

We next addressed whether increased expression of CTCF is critical to maintenance of latency I by knocking down its expression within Kem I and Mutu I cells and assessing whether this was sufficient to promote reactivation of the latency III program. As illustrated in Fig. 6B, even at a level of CTCF expression comparable to that observed in LCLs (Fig. 6B, Kem I shCTCF line 4), we did not observe detectable expression of EBNA2 or LMP1 in Kem I cells as a result. We did observe a low level of LMP1 (but not EBNA2) within the Mutu I cells, but this occurred as well within the cells that received vector encoding the control shRNA, and, as noted above, Mutu I cells are not particularly strict in their suppression of LMP1 expression. Consistent with the inability to detect EBNA2, we did not detect transcripts originating from the EBNA promoter Cp in association with knockdown of CTCF, while Qp-specific EBNA1 transcripts (indicative of latency I) were detectable in all Kem I and Mutu I lines (Fig. 6C). Finally, even after maintenance of CTCF knockdown for over a year, these cell lines continue to maintain latency I. We concluded, therefore, that either CTCF is not essential for maintenance of latency I in BL cells or a level of its expression comparable to that in B cells that support latency III is sufficient. Regardless, our results indicate that differential expression of CTCF alone is unlikely to be a significant factor in the maintenance of restricted latency once it has been established.

**CTCF contributes to establishment of restricted latency.** The above results did not exclude the possibility that CTCF contributes to silencing of the EBV genome during the early stages of infection through negative effects it may have on transcription from Cp. In support of this, Chau et al. demonstrated that in EBV-negative BL cells transfected with either a BAC DNA clone of a wt EBV genome or one mutated by deletion of the Cp CTCF site, expression of EBNA2 mRNA at 72 h posttransfection was on average 5-fold higher in the cells transfected with the mutated EBV genome (6). Though consistent with a role for CTCF in negative regulation of Cp, whether restricted latency was able to be established upon transfection with wt EBV DNA but not that of ΔCTCF EBV was not demonstrated. Moreover, it is our experience and that of others that establishment of restricted latency following infection of EBV-negative BL cells typically requires several weeks or longer (30, 50, 63). Thus, it was unclear from this previous report whether CTCF is indeed a pivotal player in the regulation of EBV latency programs through its influence on Cp.

Given these issues and the earlier-noted observation that CTCF occupancy at its site within Cp does not strictly correlate with latency type (51), we next explored the role of CTCF in the establishment of restricted latency. To do this, we generated a mutant rEBV comparable to the one previously described (6) in which the CTCF binding site of Cp was deleted. The approach that we employed to generate this mutant rEBV (ΔCTCF) from a BAC clone of the Akata EBV genome is illustrated in Fig. 7. Wild-type and ΔCTCF rEBVs (both encoding GFP and neomycin resistance) were then produced from HEK293 cells (see Materials and Methods) and used to superinfect Kem I BL cells. This B-cell model of EBV infection that we have recently developed (14) has significant

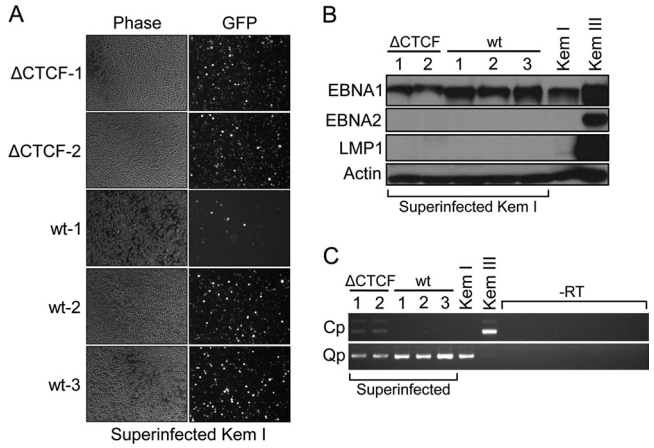


**FIG 8** Elimination of the CTCF binding site results in sustained usage of Cp. Kem I BL cells (latency I) were superinfected with either wt or ΔCTCF rEBV. (A) Detection of GFP expression indicates that superinfecting-virus genomes are retained. (B) Absence of EBNA2 and LMP1 (indicative of latency III; see Kem III positive control) suggests that CTCF is not essential for establishment of restricted latency. Detection of β-actin served as a loading control. (C) Analysis of EBNA promoter usage by RT-PCR. The low level of Cp-specific transcripts in cells superinfected with ΔCTCF but not wt rEBV suggests that CTCF is essential for efficient silencing of Cp. −RT, absence of reverse transcriptase in cDNA synthesis reaction mixture. All data shown were obtained from cells at approximately 12 months postsuperinfection.

advantages over the infection of EBV-negative BL cells to study establishment of restricted latency. Most notably, infection of EBV-negative BL lines frequently results in integration of the EBV genome (15), and the time required to convert from latency III to I can be quite variable (30, 63). In contrast, we have found that following superinfection, the frequency of genome integration is much lower or nonexistent, and the conversion from latency III to I, at both the mRNA and protein levels, is generally complete at 1 month postsuperinfection. Importantly, the EBNA gene transcriptional program of the endogenous EBV genomes within superinfected Kem I, as well as those within superinfected Akata BL cells (9), remains latency I, whereas transcription from the superinfecting-virus genomes appears to transition from latency III to I (14). Thus, establishment of restricted latency by the superinfecting virus would appear to recapitulate that which is believed to occur within normal B cells infected *in vivo*.

For these experiments, we obtained two Kem I lines from independent superinfections that stably retained infection with the ΔCTCF rEBV as demonstrated by detection of GFP expression and neomycin (G418) resistance (Fig. 8A). As shown in Fig. 8B, these lines, like those generated by superinfection with wt virus, were able to establish and maintain restricted latency as evidenced by detection of EBNA1 but not EBNA2 and LMP1. This suggested that the Cp CTCF binding site, and thus CTCF, is not essential for the establishment of restricted latency in this system. However, when we assessed EBNA promoter usage in these cells by RT-PCR, we did detect a low level of Cp activity in both Kem I lines superinfected with ΔCTCF rEBV but not in those superinfected with wt rEBV (Fig. 8C). We did note that the level of Qp EBNA1 transcripts appeared to be lower in the two lines containing ΔCTCF EBV genomes, consistent with somewhat lower EBNA1 protein levels; possible reasons for this are discussed below.

Finally, because the superinfecting strain of rEBV (Akata) has a nucleotide polymorphism in the first exon (C1) of Cp-specific transcripts (9) that we determined is not present within the endogenous (Kem) viral genome (14), we were able to confirm that these cDNAs generated from the RNA of Kem I cells superinfected with ΔCTCF rEBV did indeed originate from the superinfecting-virus genome, as expected (data not shown). Interestingly, when we reexamined Cp usage at 14 months postsuperinfection, one of the ΔCTCF lines still supported a low level of Cp usage, whereas in the other we could no longer detect Cp transcripts (data not shown). Thus, while the CTCF sites in Cp might not be absolutely essential for the establishment of restricted latency, prolonged usage of Cp in the ΔCTCF EBV genome, albeit at a level insufficient to generate detectable EBNA2, suggested that CTCF does indeed contribute to silencing of Cp.

## DISCUSSION

The mechanisms that orchestrate silencing of EBV latency gene expression during establishment of latency within B lymphocytes, and the subsequent maintenance of the protein-encoding genes in their transcriptionally inactive state (the exception being EBNA1 expression via Qp), are critical to EBV's ability to colonize its human host and thus underlie its pathogenic potential. DNA methylation has long been known to preclude EBV latency gene expression, and it is reasonable to expect that this is a regulated process, as an inappropriately directed or timed methylation would have a deleterious effect on establishment of latency. Further, an inability to sustain methylation would most likely facilitate a reactivation of viral protein expression that would subject infected B cells to detection and elimination by host T cells. One possible means to ensure maintenance of the EBV genome in its hypermethylated state is through upregulation of DNMT expression. Indeed, we consistently observed higher expression of DNMT1 and DNMT3B in B-cell lines that maintain latency I than in those that sustain latency III. Regardless, even combined knockdown of both DNMTs failed to result in reactivation of the latency III program from latency I in BL cells, suggesting that DNMT1 and DNMT3B expression comparable to that in latency III B-cell lines is, at least in the context of a previously established restricted latency, above a threshold necessary to ensure appropriate maintenance of DNA methylation-dependent transcriptional silencing.

While upregulated expression of DNMT1 and -3B does not appear to play a critical role in the maintenance of restricted latency, this may not be the case during its establishment. Previous work demonstrated that the LMP1 protein of EBV induces expression of DNMT1, -3A, and -3B when expressed within epithelial cell lines and consequently the methylation-dependent repression of E-cadherin (64). Similarly, within gastric carcinoma cell lines hypermethylation of the PTEN promoter is associated with an induction of DNMT1 expression by LMP2A (11). These results may suggest that LMP1 and LMP2A, expressed early upon infection within the latency III or II programs, may collaborate to actually initiate CpG methylation within the EBV genome through induction of cellular DNMTs.

In apparent contrast to the previous reports of LMP1 and LMP2A induction of DNMT1 and -3B, a recent report demonstrated that EBV infection of GC B cells leads to repression of DNMT1 and DNMT3B expression and an upregulation of the *de novo* DNA methyltransferase DNMT3A (28). Though repression of DNMT1 (but not DNMT3B) could be attributed to LMP1,

induction of DNMT3A expression was not due to LMP1. Moreover, the relative levels of DNMT1, -3A, and -3B observed in newly established LCLs generated from these GC B cells (which maintain latency III) matched those in cell lines derived from Hodgkin lymphoma, a tumor of GC B-cell origin (28). The apparent opposite effect of EBV on expression of DNMT1 and -3B in GC B cells (repression) and epithelial cells (activation) may reflect involvement of different cellular and/or viral factors. We note that the repression of DNMT1 and -3B in GC B cells (28) appears to be consistent with the lower level of these DNMTs in BL and LCL lines that maintain latency III relative to latency I BL lines, as observed here. Interestingly, following infection of GC B cells, DNMT3A could be detected within chromatin associated with Wp (but not Cp) (28), the EBNA promoter used prior to EBNA2 transactivation of EBNA expression from Cp. Most importantly, EBV-mediated induction of DNMT3A and its direct association with Wp, a latency gene promoter that undergoes methylation relatively early in infection (16) and which must ultimately be silenced to establish and maintain restricted latency, support the notion that methylation of the EBV genome is a regulated process. It will be interesting to determine, therefore, whether DNMT3A plays a critical role in the establishment of restricted latency beyond methylation of Wp.

Taking a similar tack to investigate the requirement for CTCF, we found that elevated expression of this multifunctional regulator of transcription is not critical for the maintenance of restricted latency in BL cells. This is perhaps not surprising given that the Cp/Wp locus is heavily methylated during latency I within BL cells, and to our knowledge there are no reported instances of direct CTCF involvement in the promotion or maintenance of DNA hypermethylation; indeed, CTCF is well known for its ability to insulate against spread of hypermethylation (38). That CTCF might be involved in maintenance of restricted latency via a negative influence on Cp was suggested previously by experiments in which transient small interfering RNA (siRNA)-mediated knockdown of CTCF in Mutu I BL cells (latency I) resulted in a small (less than 2-fold) increase in EBNA2 mRNA (presumably from Cp) (6). However, the apparent detection of small amounts of EBNA2 mRNA within Mutu I BL cells prior to knockdown of CTCF made it difficult to conclude from these results whether reduction in CTCF resulted in reactivation of Cp or simply greater transcription from copies of Cp that had undergone reactivation prior to knockdown of CTCF (as noted above, Mutu I cells are somewhat leaky in their ability to stringently maintain latency I). In contrast, upon maintenance of reduced CTCF expression in both Kem I and Mutu I BL cells over several months, even at the much lower LCL-supported levels, we failed to note any appreciable reactivation of Cp or other indicators of latency III, such as EBNA2 and LMP1 expression (Fig. 6). We conclude, therefore, that upregulation of CTCF expression (at least above that observed during latency III in LCLs) is not necessary to sustain silencing of Cp or for the maintenance of a restricted latency program in general. However, this does not necessarily preclude the involvement of CTCF, as its contribution to EBV latency may be regulated through means other than its level of expression (see below).

With respect to CTCF involvement in the initiation or establishment of restricted latency, two observations have been previously provided as evidence to support a role for CTCF, the first being a reduction in EBNA2 mRNA upon transient overexpression of CTCF in Raji BL cells (latency III) (6). The ability of CTCF to negatively regulate Cp activity, however, does not necessarily distinguish between CTCF acting as a transcriptional repressor of Cp in the classical sense, perhaps involved in the normal regulation of Cp during latency III, and CTCF as a factor in promoting the epigenetic silencing of Cp during the transition to restricted latency (though it does not necessarily preclude it from acting as such either). Second, upon transfection of EBV-negative DG75 BL and HEK293 cells with a BAC clone of either the wt EBV genome or one from which the CTCF binding site within Cp had been deleted, EBNA2 mRNA expression (presumably from Cp) was 5-fold greater from the mutated EBV genomes in both cell lines when assessed at 72 h posttransfection (6). Although both of these cell lines can support restricted latency (Cp/Wp silent) upon infection with wt EBV, this typically requires from 2 weeks (HEK293) to several months (DG75) to be established following initial support of latency III (30, 36). Thus, in the time frame of the previously described experiment (72 h), it was not possible to conclude whether loss of CTCF binding upstream of Cp would have actually precluded establishment of restricted latency, and the observed increase in EBNA2 mRNA may have simply reflected loss of normal transcriptional repression by CTCF during latency III, as noted above.

For these reasons, we generated a comparable mutant rEBV (ΔCTCF) containing the same deletion of the CTCF binding site upstream of Cp as previously described (6) and tested the ability of this virus relative to wt rEBV to establish latency I upon superinfection of Kem I BL cells. Upon superinfection with wt rEBV, these BL cells retain the endogenous viral genome in a latency I program (EBNA1 expression from Qp), and the superinfecting-virus genomes transition from latency III to I over the course of 1 to 2 months (14). Consistent with the prediction of Chau et al. (6), we found that our ΔCTCF rEBV exhibited delayed silencing of Cp, and in one of two superinfected lines obtained, Cp usage has persisted beyond 14 months. We suspect that the late silencing of Cp in the other line may have been the consequence of eventual inactivation by DNA methylation.

An important caveat was that although we observed sustained Cp activity from ΔCTCF rEBV by RT-PCR, this level of Cp usage was not sufficient for detectable expression of EBNA2 (Fig. 8B). This could not be explained by a reduction in superinfecting-virus genomes or their possible integration into host DNA, as fused-terminus analysis (42) of the EBV genomes within superinfected Kem I cells revealed that the copy numbers of superinfecting ΔCTCF and wt rEBV genomes were equivalent within their respective lines, and there was no evidence of integration through or near the viral terminal repeats (data not shown). One possible explanation may be that a latency III pattern of protein expression is not compatible with these BL cells over an extended period. Consequently, there may ultimately have been a selection for cells that supported a lower level of transcription from Cp within the superinfecting-virus genomes, i.e., below a critical threshold to circumvent deleterious effects of prolonged latency III protein expression. If so, then the fact that even low Cp usage persisted over an extended period (>12 months) despite the absence of appreciable EBNA2 (a transactivator of Cp) would appear to argue strongly that CTCF plays a pivotal role in the silencing of Cp. Additionally, we have subsequently determined that within Kem I cells superinfected with ΔCTCF virus, as well as within Kem III and Mutu III cells (positive controls for Cp usage), we can detect

transcripts initiating at least 110 bp upstream of the previously defined Cp transcription start site (data not shown). Interestingly, these adopt the splicing pattern of authentic Cp-specific EBNA transcripts (at least within their 5′ termini), though whether these are latency-specific transcripts from which the EBNAs are actually expressed is unclear at this time. While it is formally possible that they are spliced versions of read-through transcripts from an upstream lytic cycle gene, this seems unlikely given that they were not detected in an earlier nuclease protection analysis of Cp transcripts within the lytically infected (TPA-treated) B95-8 LCL (49). Further, the fact that such transcripts also were not implicated from 5′ mapping assays of Cp-derived mRNAs in latently infected cells, primarily LCLs and EBV-negative BL lines infected *in vitro* (49, 68), may indicate that Kem III and Mutu III (which came into use later) can support EBNA transcription from an alternative start site (though we have determined by RNA ligase-mediated 5′ rapid amplification of cDNA ends [RACE] that Kem III cells also support transcription from the defined Cp start site). Regardless, if these novel transcripts are not competent mRNAs for EBNA expression, this could further explain the lack of EBNA expression in the ΔCTCF EBV superinfections.

Interestingly, we also observed a lower level of EBNA1- and Qp-specific EBNA1 mRNAs in cells superinfected with ΔCTCF rEBV (Fig. 8B and C), suggesting that such a selection may have resulted in a universal reduction in EBV transcription, but it was still above a threshold necessary to produce sufficient EBNA1 for maintenance of the rEBV genome to enable cell growth under neomycin selection. While our paper was in preparation, Tempera et al., employing a chromosome conformation capture (3C) assay, reported the existence of chromatin looping within EBV genomes that is dependent on CTCF bound to its sites at Cp and Qp and which brings the EBNA1 enhancer within *oriP* in juxtaposition to Qp; deletion of the Cp binding site eliminates *oriP* association with Qp and favors Cp (57). The decrease that we observed in Qp activity as a consequence of deleting the CTCF site in Cp (Fig. 8C), therefore, would appear to be consistent with this recent report. However, it is unclear why this would have resulted in a notable decrease in overall Qp activity given the presence in our superinfected Kem I cells of endogenous (wt) genomes, the copy numbers for which are approximately an order of magnitude higher than those for the superinfecting-virus genomes (data not shown).

In summary, the results presented here together with the previous and most recent findings of Lieberman and colleagues (6, 57) are consistent with CTCF as a negative regulator of Cp that contributes to its silencing, a critical step in the establishment of persistent EBV infection. Clearly, an important issue to be resolved is how CTCF differentially orchestrates its effects on Cp. While previous (51) and current (Fig. 6) results argue against elevated expression of CTCF as a primary basis for the maintenance of restricted latency, presently we cannot exclude the possibility that increased occupancy by CTCF due to increased levels contributes to initiation of silencing of Cp. Alternatively, whether or not CTCF promotes silencing of Cp may be dependent on one or more posttranslational modifications (26, 29, 72) and/or the availability of an active cofactor(s). With respect to the latter, recent profiling of the EBV genome within Raji BL cells detected the cohesin subunit Rad21 at six sites within the EBV genome, each of which colocalized with sites of CTCF binding (13). Interestingly, two CTCF binding sites within the EBV genome with which Rad21 did not significantly associate in Raji cells (latency III) (13) were the site within Cp and that 5′ of the EBER1 gene (6, 8, 51), i.e., on the opposite side of the EBNA1-dependent enhancer of Cp within *oriP*. This may be significant insofar as cohesin may act in conjunction with CTCF in intrachromosomal looping of DNA (66), a feature that appears to be central to CTCF's function as an insulator or enhancer-blocking factor (38). Given the recent identification of CTCF-dependent alternative chromatin conformations adopted by the EBV genome in different latency programs (57), it will be interesting to determine whether differential association of cohesin with CTCF at these sites underlies CTCF's ability to silence Cp. Ultimately, it will be important to have a thorough understanding not only of how CTCF function is regulated at this locus but of how this is integrated with the DNA methylation machinery of the cell and what role EBV itself may play in regulating the transition to restricted latency.

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